Mediators of mineralocorticoid receptor-induced profibrotic inflammatory responses in the heart

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ABSTRACT

Coronary, vascular and perivascular inflammation in rats following MR (mineralocorticoid receptor) activation plus salt are well-characterized precursors for the appearance of cardiac fibrosis. Endogenous corticosterone, in the presence of the 11βHSD2 (11β hydroxysteroid dehydrogenase type 2) inhibitor CBX (carbenoxolone) plus salt, produces similar inflammatory responses and tissue remodelling via activation of MR. MR-mediated oxidative stress has previously been suggested to account for these responses. In the present study we thus postulated that when 11βHSD2 is inhibited, endogenous corticosterone bound to unprotected MR in the vessel wall may similarly increase early biomarkers of oxidative stress. Uninephrectomized rats received either DOC (deoxycorticosterone), CBX or CBX plus the MR antagonist EPL (eplerenone) together with 0.9% saline to drink for 4, 8 or 16 days. Uninephrectomized rats maintained on 0.9% saline for 8 days served as controls. After 4 days, both DOC and CBX increased both macrophage infiltration and mRNA expression of the p22phox subunit of NADPH oxidase, whereas CBX, but not DOC, increased expression of the NOX2 (gp91phox) subunit. eNOS [endothelial NOS (NO synthase)] mRNA expression significantly decreased from 4 days for both treatments, and iNOS (inducible NOS) mRNA levels increased after 16 days of DOC or CBX; co-administration of EPL inhibited all responses to CBX. The responses characterized over this time course occurred before measurable increases in cardiac hypertrophy or fibrosis. The findings of the present study support the hypothesis that endogenous corticosterone in the presence of CBX can activate vascular MR to produce both inflammatory and oxidative tissue responses well before the onset of fibrosis, that the two MR ligands induce differential but overlapping patterns of gene expression, and that elevation of NOX2 subunit levels does not appear necessary for full expression of MR-mediated inflammatory and fibrogenic responses.

INTRODUCTION

The action of mineralocorticoids has long been considered to be primarily the regulation of sodium and potassium homoeostasis via the MR (mineralocorticoid receptor) in epithelial target tissues. MR have also been identified in non-epithelial tissues such as the heart [1] and vessel wall [2], where there are now well-described (patho)physiological responses to aldosterone [3,4]. In vitro MR have an equivalent high affinity for corticosterone, cortisol and aldosterone [5], with ligand specificity for the MR conferred in vivo in epithelial target tissues.
tissues (kidney, colon and salivary glands) and the vessel wall [endothelial cells and VSMCs (vascular smooth muscle cells)] through pre-receptor inactivation of endogenous glucocorticoids (corticoids in humans, corticosterone in rats) by the enzyme 11βHSD2 (11β hydroxysteroid dehydrogenase type 2) [6,7]. This is important given that, under normal conditions, total plasma glucocorticoid levels are ≥ 1000-fold higher than aldosterone, and plasma free levels are ≥ 100-fold higher. Cardiac myocytes, however, do not express 11βHSD2 and their MR are thus normally occupied by endogenous glucocorticoids [8].

Large clinical trials have demonstrated a key role for activation of the MR in moderate-to-severe heart failure patients {RALES (Randomized Aldactone Evaluation Study) [9]} and in heart failure post-MI (myocardial infarction) {EPHESUS (Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study) [10]}. MR blockade by low-dose spironolactone or EPL (eplerenone) significantly improved patient outcomes on a number of indices, in particular in terms of survival and hospitalization. While post-hoc analysis suggests that a key contributing factor to the positive outcome is a reduction in collagen turnover, the molecular mechanisms underlying the benefits observed following MR blockade are unknown, particularly given that basal plasma aldosterone levels in both trials were normal [11]. We propose that one mechanism whereby the MR may be inappropriately activated in this context is by endogenous glucocorticoids [12].

Over the last 15 years insights into the mechanisms underlying the protective effects of MR antagonists observed in these clinical trials have been gained from studies on in vivo models of mineralocorticoid/salt-mediated cardiac fibrosis, hypertension and heart failure (reviewed in [3]). Elevated tissue fibrosis leads to cardiac dysfunction that can be characterized by reduced end-diastolic filling and reduced systolic function in both experimental animals [13] and in heart failure patients [14]. Studies in experimental models have clearly shown that mineralocorticoid administration in conjunction with a high-salt diet produces vascular inflammation and cardiac fibrosis, with tissue remodelling after 8 weeks characterized by diffuse perivascular and interstitial accumulation of collagen [15,16]. More recent studies over progressively shorter time courses (1–4 weeks) have identified the onset of tissue damage, the expression of inflammatory markers in the coronary vasculature and the recruitment of macrophages following mineralocorticoid/salt administration as key mechanisms whereby MR signalling is translated into profibrotic signals [12,17,18]. These changes have been observed as early as after 8 days of treatment and precede the onset of fibrosis, suggesting that inflammation and responses to tissue damage are requisite steps in the generation of cardiac fibrosis. This hypothesis is further supported by findings in genetically modified mice in which deletion of key inflammatory markers (osteopontin) prevents or attenuates both cardiac and renal fibrosis [19,20].

We thus proposed that when endogenous glucocorticoids activate MR, following inhibition of 11βHSD2 activity, the cardiovascular responses in terms of expression of markers of oxidative stress and vascular NO reactivity will be similar to those following exogenous mineralocorticoid administration. Accordingly, in the present study we have explored the role of endogenous glucocorticoids in mediating oxidative stress via MR over a short time course (4–16 days) with CBX (carbenoxolone) to inactivate 11βHSD2 and the selective MR antagonist EPL to confirm the MR selectivity of the responses.

MATERIALS AND METHODS

Animals

All protocols for animal use were approved by the Monash University Animal Ethics Committee. Male Sprague–Dawley rats (190–210 g starting weight) were uninephrectomized under anaesthesia with ilium xylazil (8 mg/kg of body weight; Troy Laboratories) and ketamine (60 mg/kg of body weight; Parke-Davis), with carprofen (5 mg/kg of body weight, given once subcutaneously; Pfizer) for postoperative analgesia as previously described [17]. Rats were then maintained on chow ad libitum and 0.9 % NaCl plus 0.3 % KCl solution to drink for the remainder of the study, and randomly assigned to one of ten treatment groups: (i) control, with no further treatment and killed at 8 days (CON); (ii) DOC (deoxycorticosterone; Sigma), 20 mg of DOC in corn oil given subcutaneously on day zero and killed at 4 days (DOC4); (iii) DOC as for group (ii) but killed at 8 days (DOC8); (iv) DOC, 20 mg of DOC in corn oil on day zero and day seven, and killed at 16 days (DOC16); (v) CBX at 2.5 mg of CBX·kg⁻¹ of body weight·day⁻¹ in the drinking solution and killed at 4 days (CBX4); (vi) CBX as for group (v) but killed at 8 days (CBX8); (vii) CBX as for group (v) but killed at 16 days (CBX16); (viii) CBX as for group (v) plus EPL (Pharmacia) at 100 mg of EPL·kg⁻¹ of body weight·day⁻¹ (CBX + EPL4; eplerenone was incorporated into the chow by Glenforest Stock Feeders); (ix) CBX plus EPL as for group (viii) but killed at 8 days (CBX + EPL8); and (x) CBX plus EPL as for group (viii) but killed at 16 days (CBX + EPL16).

Animals were killed by CO₂ in the air and the hearts were excised and weighed. One half was fixed in buffered 4 % (w/v) paraformaldehyde for 6 h at 4 °C, and then rinsed and stored overnight in PBS before processing for paraffin embedding. The remainder of the fresh tissue was snap-frozen in liquid nitrogen and the
RNA was extracted for RT (reverse transcription)-PCR analysis.

**Cardiac hypertrophy**

An index of cardiac hypertrophy was calculated by measuring the wet weight of the heart when the animals were killed and normalizing this to the final body weight. Values are presented as mg/g.

**Histological analysis**

Cardiac collagen content was determined by selectively staining for collagen with 0.1% Sirius Red (Sigma Diagnostics) in saturated picric acid (BDH AnalR). Digital images of systematically sampled sections were then quantified with the AIS (Analytical Imaging Station) software package (Version 4.0 Beta 1.5; Imaging Research), as previously described [21].

**Immunohistochemistry**

The inflammatory response was characterized by immunohistochemistry as previously described [17], using ED-1, the monoclonal antibody against rat monocytes/macrophages (a gift from Professor Peter Tipping, Monash University, Clayton, Australia), used at a 1:200 dilution in 1 × TBS [Tris-buffered saline; 1 × TBS is 0.137 mol/l NaCl, 0.027 mol/l KCl and 0.025 mol/l Tris/HCl]. Infiltrating ED-1-positive macrophages were quantified by counting positively stained cells in systematically sampled tissue sections. This provides a value for the average number of macrophages per frame (826890 μm²) rather than per section; more than 100 ED-1-positive macrophages were counted for each rat to allow accurate between-group comparisons.

**RT–PCR**

Total rat heart RNA was prepared with Ultraspec (Fisher Scientific). First-strand cDNA synthesis from 500 ng of total RNA was performed following DNAase treatment with AMV (avian myeloblastosis virus) reverse transcriptase (Roche) and priming with random hexamers (Promega). Quantitative PCRs were carried out using the primer sets for p22\textsuperscript{phox} (GenBank\textsuperscript{®} accession number NM_023965; forward, 5'-GAGGTCTTACTTTG-3', and reverse, 5'-CCATTCG-3'), gpt91\textsuperscript{phox} (GenBank\textsuperscript{®} accession number NM_024160; forward, 5'-ACTCCTTTATTTTTC-3', and reverse, 5'-TGCTTTGAGGAGTGCATGT-3'), gp91\textsuperscript{phox} (GenBank\textsuperscript{®} accession number NM_000274; forward, 5'-TTCCGGCTGCCACCTGAT-3', and reverse, 5'-CCATTCG-3'), eNOS [endothelial NOS (nitric oxide synthase), GenBank\textsuperscript{®} accession number V01270]. PCR amplicons were compared with gene-specific standard curves generated from 1:10 serial dilutions of previously prepared standards. Real-time PCR amplification was performed on the LightCycler (Roche) using SYBR Green reaction mix (Roche). cDNA samples were diluted 1:20 in water immediately before use for p22\textsuperscript{phox} and 18S; for all other genes, samples were analysed undiluted. Data represent the average of two separate experiments.

**Statistics**

Analysis of data were performed separately for each time point (4, 8 or 16 days) by one-way ANOVA (SPSS statistical software package, version 11.5), with Dunnett’s test to take the common control data into account; differences were considered significant at $P \leq 0.05$. An $F$ test was performed on all data sets to determine normal distribution of the data and to verify the use of Dunnett’s post-hoc test. All values are reported as means ± S.E.M.

**RESULTS**

**Cardiac hypertrophy and cardiac fibrosis**

In the present study, no cardiac hypertrophy was detected over the course of the study, consistent with the short time frame (4–16 days; results not shown). DOC administration did not produce a significant increase in cardiac collagen at 16 days (results not shown); however, collagen deposition in the CBX-treated rats at 16 days (2.14 ± 0.32%) was modestly increased ($P < 0.05$) above control (1.25 ± 0.22%), a value not different from that seen after 16 days DOC (1.37 ± 0.37%, not significant). The CBX effect was completely reversed by concomitant EPL (1.56 ± 0.84%, not significant).

**Markers of inflammation**

The cardiac inflammatory response was determined by analysis of ED-1 immunostaining and quantitative PCR for osteopontin mRNA.

**ED-1-positive macrophages**

Cardiac tissue macrophage numbers increased over time in DOC-treated rats and were above control at 4, 8 and 16 days ($P < 0.05$; Figure 1A) after administration of DOC. Macrophage infiltration in CBX-treated rats was significantly elevated ($P < 0.05$; Figure 1A) to levels equivalent to those from DOC-treated rats at the 8 and 16 day time points. Co-administration of the MR antagonist EPL with CBX significantly attenuated macrophage infiltration in rats at both 8 and 16 days ($P < 0.05$ compared with CBX alone).
Markers of inflammation

Treatment groups are as follows: CON, control; DOC, DOC treatment for 4, 8 or 16 days as indicated; CBX, CBX treatment for 4, 8 or 16 days as indicated; CBX + EPL, CBX plus EPL treatment for 4, 8 or 16 days as indicated. Values are means ± S.E.M. (n = 7 or 8, except where indicated). * P ≤ 0.05, compared with control; and † P ≤ 0.05, compared with CBX. (A) ED-1-positive macrophage recruitment. Macrophage recruitment was significantly increased at all time points for DOC or CBX treatment (n = 6 for DOC and CBX). EPL treatment returned values to baseline at 8 and 16 days. (B) Osteopontin mRNA levels. Expression of osteopontin was significantly increased by DOC at 4 and 16 days, and increased at all time points following CBX treatment. Co-treatment with EPL returned values to baseline at all time points. The mRNA levels are expressed relative to 18S RNA levels determined in parallel on the same samples.

Osteopontin gene expression

mRNA levels for osteopontin were significantly increased from 4 days after DOC administration (Figure 1B), with equivalent increases seen with CBX administration. Increases in mRNA were sustained throughout the study for both treatments. Co-administration of EPL with CBX reversed the elevated expression at each time point.

Markers of oxidative stress

Quantitative RT–PCR was performed for subunits of the NADPH oxidase complex as an indicator of changes in oxidative potential in the heart and blood vessel wall.

p22\text{phox}

The levels of p22\text{phox} increased progressively with both DOC and CBX administration. DOC increased values for p22\text{phox} mRNA at 8 and 16 days (Figure 2A; P < 0.01 compared with CON); with CBX, p22\text{phox} expression was increased after 4, 8 and 16 days of treatment (P < 0.05 compared with CON). At all time points the magnitude of response was similar for DOC and CBX treatments. The MR-selective antagonist EPL blocked the increase in mRNA levels in the CBX treatment group at 4 and 16 days (P < 0.05 compared with CBX).

NOX2 (gp91\text{phox})

A clearly different pattern of expression was seen for the macrophage/endothelial cell membrane-bound subunit NOX2 (Figure 2B). No significant increases were seen at 4 days for either treatment, whereas at 8 and 16 days only CBX produced significant increases in gp91\text{phox} mRNA levels (P < 0.05 compared with CON). These responses were blocked at both 8 and 16 days by the co-administration of EPL.

Markers of endothelial cell function and NO production

eNOS and iNOS expression were determined as an indicator of endothelium-dependent changes in vascular function, as has been shown previously [22].
Mineralocorticoid receptor and inflammation in the heart

Figure 3  eNOS and iNOS expression relative to 18S rRNA
Treatments are as for Figure 1. Values are means ± S.E.M. (n = 7 or 8 for all groups). *P < 0.05, compared with control; †P < 0.05 compared with CBX. The mRNA expression for eNOS and iNOS was normalized to 18S rRNA. (A) eNOS mRNA expression was reduced from 4 days following DOC treatment and from 8 days with CBX treatment. EPL reversed these responses. (B) iNOS mRNA expression was increased with DOC and CBX treatment at 16 days. EPL returned values for CBX to control levels. The mRNA levels are expressed relative to 18S RNA levels determined in parallel on the same samples.

eNOS
mRNA levels for the constitutively active isoform of NOS were significantly reduced following DOC administration at each time point (Figure 3A; P < 0.05 compared with CON), whereas CBX administration reduced eNOS mRNA expression only from 8 days (Figure 3A; P < 0.05 compared with CON). Co-administration of EPL partially reversed the reduced expression of eNOS at 8 days (P < 0.05 compared with CBX8) and returned values to baseline at 16 days (P < 0.05 compared with CBX16).

iNOS
mRNA levels for the inducible isoform of NOS showed a very different pattern of expression to those for eNOS. No significant differences were seen at 4 or 8 days with either DOC or CBX (Figure 3B). At 16 days, iNOS mRNA values showed an increase of similar magnitude following DOC or CBX administration (P < 0.05 compared with CON); EPL co-administration returned the elevated value to baseline (P < 0.05 compared with CBX16).

DISCUSSION

The present study shows that MR activation increases expression of markers for oxidative stress and coronary vascular inflammation in both the DOC/salt and CBX/salt rat model within 4 days. These inflammatory/oxidative stress responses precede the increases in cardiac fibrosis and hypertrophy which characterize the cardiac phenotype of these rat models at later time points. Endogenous glucocorticoid activation of MR in the presence of CBX showed molecular and cellular responses to be increased over the same time frame and, in most instances, to be of similar magnitude to those following exogenous DOC administration. These results support the hypothesis that the clinical benefits of MR blockade in heart failure patients without elevated aldosterone levels are due to spironolactone blockade of cortisol-activated MR.

MR-mediated early vascular inflammatory responses

In the present study we have demonstrated that not only is an inflammatory response induced in the coronary vasculature by both DOC and CBX, but that it is also increased above control levels after only 4 days of treatment (Figures 1A and 1B). In terms of DOC administration, infiltrating macrophage numbers were significantly and markedly increased from 4 days; although CBX treatment showed increased mean values for macrophage infiltration at earlier time points, values were significantly elevated above control only at 16 days. For osteopontin expression, DOC and CBX treatments produced significant increases from day 4. The early inflammatory response in the intramural coronary vasculature following administration of mineralocorticoids and salt is now a well-characterized prerequisite to the appearance of fibrosis at these sites [12,23], and increased infiltration of ED-1-expressing macrophages is indicative of an inflammatory response in these models. In previous studies, up-regulation of a range of inflammatory markers had been demonstrated after 1–2 weeks of treatment with mineralocorticoids plus salt, as determined by vascular ED-1-positive macrophages, COX-2 (cyclo-oxygenase-2) and the chemoattractant cytokine osteopontin [12,23]. The inflammatory response was seen at an even earlier time point than previously reported, preceding any significant increase in cardiac collagen and suggesting that inflammation is the primary response to MR activation in the vascular tree.

Although the CBX-mediated increase in macrophage infiltration only reached significance at 16 days, macrophage numbers were significantly attenuated by the selective MR antagonist EPL at both 8 and 16 days. These results are consistent with this response being induced by endogenous glucocorticoids activating MR in the vessel wall. This hypothesis is further supported by observations in a study by Ward et al. [24] where angioplasty-induced vascular damage in the porcine coronary arteries stimulated MR-mediated vascular remodelling despite normal aldosterone and salt levels. MR activation by
endogenous glucocorticoids thus appears to produce the same cardiovascular responses as DOC administration, albeit with a slightly different time course.

**MR-mediated oxidative stress**

Patients with congestive heart failure or hypertension treated with an MR antagonist indicate that aldosterone induces oxidative stress through a MR-dependent mechanism [25]. Mineralocorticoids induce oxidative stress by activation of NADPH oxidases; these changes can be blocked by concomitant MR blockade or administration of antioxidants such as apocynin [26]. A number of mechanisms have been proposed for aldosterone-mediated oxidative stress including increased oxidized LDLs (low-density lipoproteins) promoting activation of vascular NADPH oxidase, changes in NO and PP2A (protein phosphatase 2A) intracellular signalling pathways [27], and eNOS signalling [28,29]. Endothelial dysfunction contributes to the development of elevated blood pressure, vascular remodelling and tissue fibrosis in response to mineralocorticoids [30,31]. Of relevance to the present study, endothelial dysfunction and oxidative stress have also been proposed as a mechanism contributing to hypertension and diminished renal function following 11βHSD2 deficiency or blockade [32].

The present study extends the findings of Sun et al. [26], who demonstrated an increase in NADPH oxidase expression from week 4 of treatment by aldosterone, in that our results demonstrate a doubling in expression of the p22\textsuperscript{phox} subunit of NADPH oxidase after only 4 days of DOC or CBX treatment. Increases in p22\textsuperscript{phox} expression have been previously shown to parallel increases in ROS (reactive oxygen species) production [33–35]. The effects of CBX plus salt were blocked by the selective MR antagonist EP1, demonstrating that these effects are MR-specific. In contrast, mRNA levels for the NOX2 (gp91\textsuperscript{phox}) isoform of NOX1/NOX4 showed a very different pattern of expression. CBX treatment alone at 8 and 16 days induced NOX2 expression, which is largely expressed in endothelial cells and macrophages, whereas DOC did not. Previous studies, over much longer time courses (4–8 weeks), have shown up-regulation of NOX2 by DOC administration. Given that it is not up-regulated at 4–16 days, the later increase is unlikely to be a primary gene response. Increased expression of the components of the NADPH oxidase system in 4–8 week studies support the importance of this source of reactive oxygen in the ongoing tissue damage and inflammatory processes. Indeed, up-regulation of NOX2 mRNA by CBX in the present study may not reflect primary gene transcription, but nevertheless remains clear evidence for ligand-specific MR-mediated gene expression, i.e. aldosterone compared with cortisol/corticosterone. The concept of distinct gene expression profiles with different ligands acting at the same receptor has been well-described for other members of the steroid hormone receptor family and is the basis of the development of selective modulators of these receptors [36,37]. Further studies with a time course of 1–2 h are necessary to determine whether the p22\textsuperscript{phox} subunit of NADPH oxidase is a primary MR response gene and whether other isoforms of the NADPH oxidase subunits may be regulated in a ligand-specific manner.

It remains unclear in this model whether oxidative stress promotes inflammation or inflammation is the source of oxidative stress, or both. The initiation of tissue inflammatory responses, specifically recruitment and activation of macrophages, requires an increase in cellular stress or damage which leads to expression of inflammatory chemokines and other micro-environmental signals by the epithelium [38,39]. A significant contributory role for ROS and oxidative stress (concomitant with NADPH oxidase expression) to the inflammatory response following aldosterone administration has been suggested previously [26,40]. NADPH oxidase production of superoxide in response to inflammatory cytokines, such as TNF-α (tumour necrosis factor-α), IL-1β (interleukin-1β) and interferon-γ, has been demonstrated to be a major source of ROS generation, secondary to the induction of an inflammatory response, in the blood vessel wall [36–38]. Other cellular mechanisms which are potential sources of ROS generation and may be stimulated in this model are the mitochondrial electron-transport chain and xanthine oxidase [41].

**Markers of NO signalling**

We have shown that DOC plus salt administration in vivo is sufficient to reduce eNOS mRNA levels at 4 days, whereas CBX treatment produced a significant reduction at 8 days (Figure 3A). The response to CBX was less at 8 days and returned to baseline after 16 days of EPL co-administration, again indicating the contribution of the MR and suggesting variation in the time course of the response. This is not the first demonstration of a role for MR in the regulation of eNOS and eNOS-dependent vascular reactivity. Nagata et al. [42] have shown that 10⁻⁷ M aldosterone significantly reduced eNOS expression and NO production in HUVECs (human umbilical-vein endothelial cells) after 16 h of treatment; this was reversed by MR blockade or by blockade of the oxidative stress responses. Similarly, reduced eNOS expression in SHRs (spontaneous hypertensive rats) can be reversed by the administration of EPL [43], a response which correlated with improved vascular reactivity in the treated rats. CBX specifically affects vascular endothelial cells, and not VSMCs, when administered in vivo, with changes in endothelial cell NO signalling and endothelial-mediated vascular relaxation key mechanisms whereby CBX treatment can affect systolic blood pressure [44]; our reduced expression of eNOS in the treatment groups is consistent with these physiological changes. Although vascular reactivity was not directly tested in the present study, our findings are consistent with previous studies and suggest...
that similar mechanisms of vascular dysfunction are operating. Blockade of 11βHSD2 has also been correlated with changes in eNOS signalling and endothelial cell dysfunction. Consistent with results from the present study, Quasching et al. [32] showed that, following glyccheryretinic acid administration to rats, renal eNOS and renal function were reduced, and blood pressure was elevated. These changes were reversed by spironolactone, albeit over a longer time course than that used in the present study. In contrast, iNOS showed a different pattern of expression, with mRNA levels increasing after 16 days with either treatment, again evidence for a secondary effect. These responses are clearly MR-activation-dependent, in that they are blocked by EPL; the time course, however, suggests that it is a response evoked downstream of the primary responses to MR activation.

Inappropriate activation of MR by glucocorticoids

There are three circumstances by which MR can be potentially inappropriately activated in vivo: (i) administration of exogenous DOC or aldosterone plus salt [18,23,45]; (ii) administration of 11βHSD enzyme inhibitor CBX plus salt to intact rats, allowing endogenous corticosterone to activate the MR [12,45]; and (iii) tissue damage (accompanied by ROS generation)-induced MR-mediated vascular remodelling as seen after experimental angioplasty [24]. The present study confirms the ability of endogenous glucocorticoids to activate MR and produce pathophysiological effects when 11βHSD is inhibited. In angioplasty studies, no CBX, DOC or salt were administered and circulating steroid levels were normal, suggesting that some mechanism, related to the initial vascular damage, allows endogenous glucocorticoids to occupy and activate the VSMC MR. These observations imply the involvement of MR activation by endogenous glucocorticoids in cardiovascular disease associated with vascular damage or inflammation, and provide a rationale for the clinical use of MR antagonists in heart failure despite normal aldosterone levels.

We have not measured plasma or tissue levels of corticosterone in the present study. However we have previously shown that inhibiting CBX does not increase plasma corticosterone levels at 8 days (M. J. Young, J. Morgan and A. Rickard, unpublished work) while other investigators have shown that rats treated for 3 weeks and mice for 2 weeks with CBX show no significant increase in plasma corticosterone [46,47]. For CBX regulation of tissue levels of corticosterone, Usa et al. [48] showed modest increases in local renal corticosterone levels in response to direct infusion of CBX to the renal medulla, whereas plasma levels did not change. Although modest changes in the concentration of corticosterone available to bind the MR in the present study appears to be possible, it is unlikely to explain the increase in MR activation in the present model.

Conclusions

With regard to our current knowledge of the field, these results support the working hypothesis that many of the tissue responses to DOC-salt and CBX-salt appear to be indistinguishable [12], and are evidence for the general commonality of MR-induced gene expression whether the agonist ligand is a classic mineralocorticoid or corticosterone when 11βHSD2 is blocked. The clear difference between ligands for NOX2 induction is unexpected and demonstrates that an increase in NOX2 expression is not required for what appears to be the initiation of a full tissue inflammatory response to MR activation. It follows that if this is the case then membrane-bound NOX2 is not rate-limiting, with basal levels of this component of the NADPH oxidase system sufficient for enhanced activity of the complex as a whole. It is also possible that the differences in NOX2 gene expression that we observed for the different MR ligands may also reflect tissue-specific gene transcription, given that NOX is restricted to endothelial cells and macrophages.

FUNDING

This work was supported by the National Health and Medical Research Council (NHMRC) of Australia [grant number 388914].

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